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COMBINED TRANSMITTAL OF APPEAL BRIEF TO THE BOARD OF PATENT APPEALS AND INTERFERENCES & PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. 1.136(a) (Large Entity)	Docket No. GA0118USC

In Re Application Of: **Nicolette, Charles A.**

Application No.	Filing Date	Examiner	Customer No.	Group Art Unit	Confirmation No.
10/041,977	January 9, 2002	Padmashi Ponnaluri	24536	1639	7476

Invention: **A METHOD FOR IDENTIFYING CYTOTOXIC T-CELL EPITOPES**

COMMISSIONER FOR PATENTS:

This is a combined Transmittal of Appeal Brief to the Board of Patent Appeals and Interferences and petition under the provisions of 37 CFR 1.136(a) to extend the period for filing an Appeal Brief.

Applicant(s) hereby request(s) an extension of time of (check desired time period):

☐ One month ☐ Two months ☐ Three months ☐ Four months ☒ Five months

from: **September 19, 2005** (*Date*) until: **February 19, 2006** (*Date*)

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Fee for Appeal Brief:	$500.00
Fee for Extension of Time:	$2,160.00
TOTAL FEE FOR APPEAL BRIEF AND EXTENSION OF TIME:	**$2,660.00**

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☒ Please charge Deposit Account No. **07-1074** in the amount of **$2,660.00**

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10/041,977	January 9, 2002	Padmashi Ponnaluri	24536	1639	7476

Invention: **A METHOD FOR IDENTIFYING CYTOTOXIC T-CELL EPITOPES**

TO THE COMMISSIONER FOR PATENTS:

This combined Transmittal of Appeal Brief to the Board of Patent Appeals and Interferences and petition for extension of time under 37 CFR 1.136(a) is respectfully submitted by the undersigned:

Jennifer D. Tousignant

Signature

Dated: 2/21/2006

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FEE TRANSMITTAL for FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) $2,660.00

Complete if Known	
Application Number	10/041,977
Filing Date	January 9, 2002
First Named Inventor	NICOLETTE, Charles A.
Examiner Name	Padmashi Ponnaluri
Art Unit	1639
Attorney Docket No.	GA0118USC

METHOD OF PAYMENT (check all that apply)

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		Extra Claims	Fee ($)		Fee Paid ($)
_____	- 20 or HP =	_____	x $50.00	=	$0.00

HP = highest number of total claims paid for, if greater than 20.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	_____

Indep. Claims		Extra Claims	Fee ($)		Fee Paid ($)
_____	- 3 or HP =	_____	x $200.00	=	$0.00

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

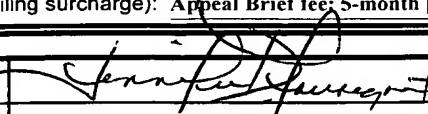
If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listing under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		Extra Sheets		Number of each additional 50 or fraction thereof		Fee ($)		Fee Paid ($)
_____	- 100 =	0	/ 50	0	(round up to a whole	x $250.00	=	$0.00

4. OTHER FEE(S)

	Fee Paid ($)
Non-English specification, $130 fee (no small entity discount)	
Other (e.g., late filing surcharge): Appeal Brief fee; 5-month petition	$2,660.00

SUBMITTED BY

Signature		Registration No. (Attorney/Agent)	54,498	Telephone	508.270.2499
Name (Print/Type)	Jennifer D. Tousignant			Date	2/21/2006

This collection of information is required by 37 CFR 1.136. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Patent
Our Docket: GA0118USC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: NICOLETTE, Charles	)	Art Unit: 1639
Serial No. 10/041,977	)	Examiner: Padmashri Ponnaluri
Filed: January 9, 2002	)	
For: A method for identifying cytotoxic T-cell epitopes	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST-CLASS MAIL IN AN ENVELOPE ADDRESSED TO: COMMISSIONER FOR PATENTS, P.O. BOX 1450, ALEXANDRIA, VIRGINIA 22313-1450

Date **Signature of person mailing correspondence**

Appeal Brief under 37 C.F.R. § 41.37

This Appeal Brief is being filed pursuant to a Notice of Appeal filed on July 19, 2005 in connection with the above referenced patent application. This Appeal Brief was originally due on September 19, 2005. As part of this communication, Applicant is filing a Petition for a Five Month Extension of Time, thereby extending the deadline to file this Appeal Brief to February 19, 2006. February 19, 2006 falls on a Sunday while the next business day is Monday, February 20, 2006, which is a federal holiday. Accordingly, the next business day on which to file this Appeal Brief is Tuesday, February 21, 2006. Accordingly, this response is timely filed.

TABLE OF CONTENTS:

A. REAL PARTY IN INTEREST

The real party in interest is Genzyme Corporation, the assignee of record.

B. RELATED APPEALS AND INTERFERENCES

There are no related appeal and interferences known to Appellant, which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

C. STATUS OF CLAIMS

Claims 1-18, 20-23, and 25-28 are currently pending.

Claims 19 and 24 are canceled.

Claim 3 stands withdrawn as being drawn to a non-elected species.

Claims 1-2, 4-18, 20-23, and 25-28 stand rejected, said rejections are those currently under appeal.

D. STATUS OF AMENDMENTS

No amendments were filed subsequent to the final rejection of the instant claims.

E. SUMMARY OF CLAIMED SUBJECT MATTER

There is one independent claim present in the claims under appeal, which is claim 1. The present invention as claimed in independent claim 1 provides a method for screening an oligopeptide library for bioactive cytotoxic T lymphocyte (CTL) epitopes. The oligopeptide library contains a conserved structural motif corresponding to a structural motif characteristic of peptides that associate with the MHC-haplotype to which the cytotoxic T cells are restricted. This structural motif is referred to in the art as an agretope and is the portion of the oliogopeptide that interacts with the MHC molecule.

In this method, (a) the cytotoxic T cells all share the same MHC-haplotype restriction ("a population of cytotoxic T cells having the same MHC-haplotype restriction"), (b) the released molecules or peptides all come from a library based upon the MHC-haplotype restriction of those cytotoxic T cells ("contains a structural motif corresponding to an agretope of the MHC-haplotype to which said cytotoxic T cells are restricted"), and (c) the antigen presentation means is also based upon the MHC-haplotype of the cytotoxic T cells ("which antigen presentation means correspond to the MHC-haplotype to which the cytotoxic T cells are restricted"). The correlated cytotoxic T cells, library of molecules and the antigen presentation means permits complete testing of a less complex library with the goal of finding a range of active molecules, including but not limited to the native sequence. (Supported throughout the instant specification, and, particularly at paragraph [0032[and [0087] - [0097].]

F. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Whether claims 1-2, 4-7, 9-17, 20-23, and 25-28 are unpatentable under 35 U.S.C. §103(a) over Van der Zee et al. (European Immunology, 1989, Vol.19: 43-47) and Lam et al. (U.S. Patent Serial No. 5,510,240)?

2. Whether claims 1-2, 4-18, 20-23, and 25-28 are unpatentable under 35 U.S.C. §103(a) over Van der Zee et al. (European Immunology, 1989, Vol.19: 43-47) and Lam et al. (U.S. Patent Serial No. 5,510,240) and further in view of Englehard (Current Opinion in Immunology, 1994, Vol. 6: 13-23?

3. Whether claims 1-2, 4-7, 9-18 20-23, and 25-28 are unpatentable under 35 U.S.C. §103(a) over Van der Zee et al. (European Immunology, 1989, Vol.19: 43-47) and Lam et al. (U.S. Patent Serial No. 5,510,240) and further in view of Melief et al. (U.S. Patent Serial No. 5,554,724)?

G. ARGUMENT

Errors in the Rejection under 35 U.S.C. 103(a) of claims 1-2, 4-18, 20-23, and 25-28 over Van der Zee et al. (European Immunology, 1989, Vol.19: 43-47) in combination with one or more other secondary and tertiary references (Lam et al., US Pat No. 5,510,240; Englehard, *Current Opin. Immunol.* 1994 6:13-23; Melief et al., US Pat No. 5,554,724).

1. Brief summary of the applied references

i. Van der Zee et al. (European Immunology, 1989, Vol.19: 43-47).

Van der Zee teaches a modification of the "Pepscan" method and the use of said modified method to delineate a T cell epitope for a known antigen. The original Pepscan method, published in 1984[1], provided a method to simultaneously synthesize hundreds of individual peptides onto solid supports. Each solid support held an individual peptide and the solid supports were arranged in a microtiter plate format. In this format, the interaction of an antibody with each individual peptide was measured without removing the peptide from the support. This enabled the rapid analysis of peptide antigen-antibody interactions.

However, the Pepscan technology was not immediately applicable to the study of peptide-T-cell interactions. In order to study these interactions, the peptide could not be attached to a solid support since the peptide has to be presented to the T-cell in the context of an MHC molecule on an antigen-presenting cell (APC) for interactions to occur. The peptide must be detached from the solid support in order for the APC to present the peptide. Van der Zee overcame this Pepscan limitation by modifying the existing Pepscan technology. The modification made the peptide available from the solid support. Van der Zee accomplished this via chemical cleavage (formic acid), enzymatic cleavage (trypsin digestion), or physical cleavage (filing of the polyethylene rod).

ii. Lam et al. (U.S. Patent Serial No. 5,510,240) hereinafter referred to as Lam.

Lam discloses methods to generate a bio-oligomer library where each individual bio-oligomer species is attached to a solid phase support. The individual library species are generated by sequentially coupling random monomer subunit sequences together. Therefore, all potential combinations of monomer subunits are included in the library of oligomers. In one embodiment, the subunit(s) may be amino acids, amino acid analogues, or peptidomimetics.

iii. Englehard, V.H., 1994, Curr. Opin. Immunol., Vol. 6: 13-23.

Englehard is a review in the field of immunology. It reviews progress in the field that relates to the structure of MHC class I molecules and the structure of peptides that bind to and interact with MHC class I molecules.

iv. Melief et al. (U.S. Patent Serial No. 5,510,240) hereinafter referred to as Melief.

Melief discloses peptides derived from the MAGE-2 tumor rejection antigen precursor. In identifying specific peptides, Melief utilized a certain cell line, 174CEM.T2, to determine whether a particular peptide was capable of binding to a specific MHC molecule, HLA-A2.1.

2. Claims 1-2, 4-18, 20-23, and 25-28 are unobvious over the disclosures in the cited references. The Graham factual inquiries were not correctly applied in the determination of obviousness under 35 U.S.C. § 103. Hence, no *prima facie* case of obviousness has been or can be established by the Office.

Claims 1-2, 4-18, 20-23, and 25-28 stand finally rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Van der Zee et al., *Eur.J.Immunol.* 19:43-47 (1989) in combination with one or more other secondary and tertiary references (Lam et al., US Pat No. 5,510,240; Englehard, *Current Opin. Immunol.* 1994 6:13-23; Melief et al., US Pat No. 5,554,724). Appellant respectfully traverses.

As stated in the MPEP 2141, it is Office policy to follow Graham v. John Deere Co., 383 U.S. 1, (1966) in the determination of obviousness under 35 U.S.C. 103. This requires determining the scope and content of the prior art, the differences between the prior art and the claimed invention, and the level

[1] Geysen et al., 1984, Proc. Natl. Acad. Sci. USA, Vol. 81: 3998-4002, provided herein in the Evidence Appendix.

of ordinary skill in the art. Office policy states that the following basic tenets, which follow from Graham, must be applied during the obviousness analysis (MPEP 2141). These tenets were listed by the Federal Circuit in Hodosh v. Block Drug Co., Inc., 229 USPQ 182, (Fed. Cir. 1986) as follows: 1) The claimed invention must be considered as a whole; 2) the references must be viewed without the benefit of impermissible hindsight vision afforded by the claimed invention; and 3) the reasonable expectation of success is the standard against which obviousness is determined not the "ought to be tried" standard.

Appellant asserts that the Office has erred in rejecting the instant claims by not adhering to these basic tenets in the application of 35 U.S.C. 103.

Furthermore, as stated in MPEP 2142, the Office bears the initial, legal burden of establishing a *prima facie* case of obviousness in accordance with the Graham factual inquiries. The establishment of this *prima facie* case requires three basic criteria. MPEP 2142-2143 outlines these criteria, which the Federal Circuit has consistently required in establishing obviousness where references are combined. There 1) must be a teaching, motivation, or suggestion shown by the Examiner to combine the cited references[2]; 2) all claim limitations must be taught or suggested by the references[3]; and 3) there must be a reasonable expectation of success in making the combination of the references[4].

Appellant asserts that the Office has failed to meet its burden in establishing a prima facie case of obviousness. The Office has failed to properly establish all three criteria in the instant rejection. Moreover, Appellant notes that the Federal Circuit has rejected the establishment of a prima facie case of obviousness where even a single criterion was absent[5].

---

[2] *In re* Rouffet, 47 USPQ 2d 1453, 1457-1458 (Fed. Cir. 1998). "...this court requires the examiner to show a motivation to combine the references that create the case of obviousness. In other words, the examiner must show reasons that the skilled artisan, confronted with the same problems as the inventor and with no knowledge of the claimed invention, would select the elements from the cited prior art references for combination in the manner claimed."

[3] *In re* Gulack, 703 F.2d 1381, 1385 (Fed. Cir. 1983). "The 1952 act legislatively revised that approach through its requirement that the claim be viewed as a whole in determining obviousness. *Graham v. John Deere Co., 383 U.S. 1, 148 U.S.P.Q. (BNA) 459, 15 L. Ed. 2d 545, 86 S. Ct. 684 (1966).* The CCPA has considered all of the limitations of the claims, including the printed matter limitations, in determining whether the invention would have been obvious. See *In re Royka, 490 F.2d 981, 180 U.S.P.Q. (BNA) 580 (CCPA 1974); In re Cavrich, 59 C.C.P.A. 883, 451 F.2d 1091, 172 U.S.P.Q. (BNA) 121 (1971).*

[4] *In re* Vaeck, 947 F.2d 488, 493 (Fed. Cir. 1991). "Where claimed subject matter has been rejected as obvious in view of a combination of prior art references, a proper analysis under § 103 requires, inter alia, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art [**16] that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. Both the suggestion and reasonable expectation of success must be founded in the prior art, not in the applicant's disclosure." (Citations omitted).

[5] *In re* Rouffet, 47 USPQ 2d 1453 at 1458, (Fed. Cir. 1998). "Lacking a motivation to combine references, the Board did not show a proper prima facie case of obviousness."

a. The Office did not consider the claimed invention as a whole.

When ascertaining the differences between the claimed invention and the prior art, the claimed invention must be considered as a whole. It is impermissible to reduce the claimed invention down to an inventive concept or "thrust" that disregards the invention in its entirety. All claim limitations must be considered. W.L. Gore v. Garlock, Inc., 721 F.2d 1540, 1548, (Fed. Cir. 1983). The Office has erred in finding the instant invention obvious because it distilled the claimed invention down to a "gist" and disregarded all the limitations claimed.

Rather than examining the instant invention with all its limitations, the Office has reduced the instant invention to the use of peptide libraries to screen for T cell epitopes. No reference to a critical limitation of the instant invention, wherein each of the assay components is correlated for MHC-haplotype status and including the use of peptide libraries based upon MHC-haplotype status of the population of cytotoxic T cells to be tested, appears in the rejections.

The Office's distillation impermissibly disregards this express feature of the instant invention. By focusing on the general use of peptide libraries to screen for T cell epitopes, the Office has not evaluated Appellant's non-obvious finding that using assay components correlated for MHC-haplotype status permits complete testing of a less complex library with the goal of finding a range of active molecules, including but not limited to the native sequence.

b. The references were combined impermissibly with the benefit of hindsight vision afforded by the instant invention.

The Office fails to provide a detailed statement of motivation, which set forth the requisite basis for combining the references. Moreover, when the cited references are examined more closely, it is readily appreciated that they teach away from the instantly claimed invention.

(i) The required teaching, motivation, or suggestion to combine the cited references has not been identified by the Office.

The Office's rejection is primarily directed at identifying the various elements that the Office concludes may be combined to arrive at the instant invention. The detailed statement of motivation to provide a basis for combining the references is absent. Rather, the instant rejections contains only conclusory statements to address the motivation behind combining the cited references, as cited below:

> Thus, one skilled in the art at the time the invention was made, motivated to use methods of Lam et al. in the methods of Van der Zee with the expectation of identifying T cell epitopes and determine the structure of the epitopes and use the information in synthesis of T cell epitope variants which would be useful as therapeutics or in diagnosis. Office Action mailed April 21, 2004 at p. 8, lines 14-18.
>
> Thus, it would have been obvious to one skilled in the art at the time the invention was made to use the motifs disclosed by Englehard et al in the methods of Van der Zee et al, and Lam et al with the expectation of obtaining new T cell epitopes which would bind higher affinity. And using the methods of Van der Zee et al and Lam et al to synthesize a larger number of peptides simultaneously and screen for higher affinity T cell epitope and determining the structure of the peptide. Office Action mailed April 21, 2004 at p. 9, line 21 -p. 10, line 4.
>
> Thus, it would have been obvious to one skilled in the art at the time the invention was made to ude 174xCEM.T2 cell line disclosed by Melief et al in the method of Van der Zee and Lam et al with the expectation of identifying high affinity T cell epitopes and with the expectation of using them as immunotherapeutics. Office Action mailed April 21, 2004 at p. 10, lines 17-20.

These statements invoke the knowledge of an artisan as the source for a motivation to combine the references. However, it completely lacks the factual evidence necessary to support this contention. The Office's statement itself cannot suffice as evidence[6]. Moreover, the Office fails to identify the specific rationale or principle known by the hypothetical artisan that would have motivated the combination. When an Office relies on the knowledge of a hypothetical artisan in making an obviousness rejection, this specific rationale, principle, or line of reasoning is required[7]. The Office's rejections above clearly lack a specific, motivating rationale or principle. Appellant asserts they are non-specific and unsupported. Certainly more is required by the Office.

The cited references themselves demonstrate the lack of adequate motivation for the combination set forth by the Office. Contrary to the Office's conclusion, one of skill in the art would not be motivated to combine the libraries comprising the selectively cleavable linkers of Lam et al. in the method of Van der Zee et al.

First, Van der Zee et al. already discloses means for detaching (i.e., cleaving) peptide from the supports.[8] Appellant wonders why an artisan would seek an alternative cleavage method where a known, successful method was provided within the reference.

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[6] *In re* Dembiczak, 50 USPQ 2d 1614, 1617 (Fed. Cir. 1999), *abrogated on other grounds.* "Broad conclusory statements regarding the teaching of multiple references, standing alone, are not 'evidence'."

[7] *In re* Rouffet, 47 USPQ 2d 1453 at 1458 (Fed. Cir. 1998). "...must identify specifically the principle, known to one of ordinary skill, that suggests the claimed combination" and "...must explain the reasons one of ordinary skill in the art would have been motivated to select the references and to combine them to render the claimed invention obvious."

[8] See, Van der Zee et al., at page 43, abstract, lines 11-13; at page 43, col.2 "in the present study we developed a procedure for the detachment of the peptide from the solid phase under mild conditions"; at pages 44-45, section 2.1 and section 3.1; at pages 46-47, section 4.

Second, Van der Zee et al. admits that the quantity or amount of peptide cleaved from the supports: (i) are adequate (enabling analysis) for several experiments; and (ii) correspond to about 10% of the amount present on the supports before cleavage (i.e., leaving peptide on the support available for further analysis).[9]

Accordingly, if one of ordinary skill in the art could achieve cleavage of a quantity of peptide from the support adequate for analysis while also retaining a quantity of that peptide on the support, available for additional analysis, as disclosed in Van der Zee et al., the logic supporting the asserted "motivation" (Office Action mailed April 21, 2004 at p. 8, reproduced above) clearly fails. Therefore, the asserted "advantages" of using selectively cleavable linkers (Office Action mailed April 21, 2004 at p.8, lines 7-12)

> such that only a fraction of peptides are cleaved from the beads to identify T cell epitopes taught by Van der Zee et al. and still have peptides attached to the beads which would be useful in structure analysis methods . . .

are moot, because those alleged "advantages" are clearly taught in the method of the Van der Zee et al. reference.

Furthermore, if one of ordinary skill in the art did combine the selectively cleavable linkers of Lam et al. in the method of Van der Zee et al., such combination would neither teach nor suggest the claimed invention. As discussed above, the "combination" would not place the artisan in a better position than the artisan would have if employing only the method of the primary reference.

Given the clear failure of Van der Zee et al. (taken alone or when combined with Lam et al.), as discussed hereinabove, it unmistakably follows there is no motivation to combine with Engelhard, or for a combination with Melief et al.

(ii) <u>The primary reference teach away from the suggested combinations</u>

Appellant notes that Van der Zee et al. teaches away from the presently claimed invention for a variety of reasons:

Firstly, none of Van der Zee's derivatives were significantly superior to the native epitope. Therefore, other than for mapping or characterization of a known T cell epitope, one skilled in the art would not be motivated to prepare or analyze derivative epitopes.

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[9] See, footnote 6, and Van der Zee et al. at page 45, col.2, lines 6-11; at page 47, col.2, lines 1-4.

Review of the data presented by Van der Zee in Tables 2, 3 and 4 readily demonstrates that no derivatives showed significantly enhanced ability to stimulate T cell activity. Moreover, Van der Zee notes that the majority of residues was absolutely required for activity[10]. They cite work by other researchers finding that replacement of certain residues can result in equal or higher stimulatory activity; but, given Van der Zee's data, the authors conclude "indifferent residues is not a general feature of T cell epitopes." It seems clear that Van der Zee makes the case that the method is useful for mapping and characterization, but not more.

Secondly, all derivatives tested by Van der Zee were based upon the known native sequence. When different than the native, such derivative contained only one change per peptide. Therefore, if one skilled in the art sought to make and test derivative epitopes according to Van der Zee, the native epitope would be used as a template with only single residue changes.

There is no reason to expend the significant time and energy to create libraries of peptides sharing the same MHC-haplotype based upon Van der Zee. Van der Zee's teaching would not encourage others to expend time or resources in a broad search for non-native epitopes. It is clear that a disciple of Van der Zee would to look skeptically at the time and energy spent in creating a library of derivatized natural epitopes based upon any criteria other than based upon a known sequence.

As discussed above, if one has identified the natural epitope according to Van der Zee, there is no reason to look further. Contrary to the conventional teaching at the time the present invention was filed, however, the instant inventor is actively seeking a method to identify a wide range of "derivatized natural epitopes" (i.e., non-natural or altered ligands). The inventor has determined that non-native ligands offer improved immunological reactivity and hence, developed his method to identify each molecule or peptide specie that elicits the cytotoxic T cell response in a given library.

Notwithstanding the efforts to date to identify T cell epitopes, the inventor has recognized a clear need in the art for a rapid method to identify cytotoxic T cell epitopes. In several cases, derivatized natural epitopes are more effective than the natural epitope itself, accordingly, there is a need to identify such derivatized natural epitopes.

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[10] "In other studies investigating single amino acid substitutions at all positions within a defined sequence critical for proliferation, the replacement of some residues resulted in equal or even higher stimulatory activity...The present study shows that the occurrence of such indifferent residues is not a general feature of T cell epitopes. Apparently, all residues in the relatively short 180-186 sequence are required for association either with the MHC molecules, or with the T cell receptor or with both." (Van der Zee at page 47, col. 1).

c. The cited references are not combinable even with the benefit of impermissible reliance on the claimed invention because not all elements of the instant invention are taught in the cited references

1) Van der Zee does not teach the use of T cells, oligopeptides and antigen presenting means, each of which correspond to the same MHC-haplotype restriction.

Applicant respectfully asserts that, neither Van der Zee, nor any of the cited secondary references, teach or suggest any method to identify cytotoxic T cell epitopes wherein each of the assay components is correlated for MHC-haplotype status. By the present invention, (a) the cytotoxic T cells all share the same MHC-haplotype restriction ("a population of cytotoxic T cells having the same MHC-haplotype restriction"), (b) the released molecules or peptides all come from a library based upon the MHC-haplotype restriction of those cytotoxic T cells ("contains a structural motif corresponding to an agretope of the MHC-haplotype to which said cytotoxic T cells are restricted"), and (c) the antigen presentation means is also based upon the MHC-haplotype of the cytotoxic T cells ("which antigen presentation means correspond to the MHC-haplotype to which the cytotoxic T cells are restricted"). The correlated cytotoxic T cells, library of molecules and the antigen presentation means permits complete testing of a less complex library with the goal of finding a range of active molecules, including but not limited to the native sequence.

Since "a population of cytotoxic T cells" of the instant invention have the "same MHC-haplotype," only one MHC-haplotype is defined at a time. Since each MHC-haplotype is defined by a different structure, a different peptide library will be used for each agretope of the MHC-haplotype. In this manner, each released oligopeptide will correspond to the same agretope of the MHC-haplotype (and to which the cytotoxic T cells are restricted).

This concept of using peptide libraries based upon MHC-haplotype status of the population of cytotoxic T cells to be tested is not found in Van der Zee. The present invention offers advantages of reducing complexity of the library, while at the same time preserving a potential of finding every reactive member of the library if desired. '

This goal of seeking enumeration of multiple peptides capable of eliciting a cytotoxic response when complexed with a cytotoxic T cell and antigen presentation means is also not found in the prior art. According to the teachings of Van der Zee, it would not be useful to create degenerate libraries

conserved only for MHC-haplotype because, according to Van der Zee, all native residues are seen as essential[11].

Moreover, since Van der Zee's assay relies upon syngeneic thymocytes as antigen presentation means (see, Van der Zee at Section 2.2), it would not be necessary for Van der Zee to correlate the MHC status of the cytotoxic T cells, the oligopeptides and the antigen presentation means. By definition, a syngeneic system is from the same organism.[12] Thus, syngeneic thymocytes have a repertoire of all the necessary antigen presentation means for each T cell tested, and therefore, need not be matched for MHC-haplotype. Distinguishable from the aforementioned syngeneic system, is a scenario where one contacts a population of cytotoxic T cells with antigen presenting cells displaying peptides that are not correlated for MHC haplotype. In this scenario, cytotoxic T cells will not recognize the antigen presenting cell:peptide complexes and, thus, only random, fortuitous T cell activity will be detected.

Van der Zee created several limited libraries; all closely based upon the native sequence of the 65-kDa mycobacterial protein, and, in fact, only modified one residue at a time. Specifically, Van der Zee tested 28 overlapping peptides for mapping of the native epitope (see, Van der Zee at Table 2), and, for characterization of the necessary residues an additional nine variants containing a single alanine substitution per peptide (see Van der Zee at Table 3) together with several deletion peptides (see Van der Zee at page 46, col. 2). Van der Zee found that all residues of the minimal epitope and seven of the nine residues (of the full epitope) were essential for stimulation of the T cell clones and noted that each of these residues were required. (See, Van der Zee at page 47, first paragraph). Thus, if one were to make libraries based upon the teachings of Van der Zee, they would contain only one difference per peptide as compared with the native antigen. Van der Zee uses degenerately designed sequences to sequentially study the contribution of each native residue.

Van der Zee goes on to comment on work done by other researchers in the field, also using single amino acid substitutions, which found that modification of certain residues could result in higher activity. Taking Van der Zee's data in view of these other researchers, at page 47, Van der Zee still concludes that all residues of the native epitope sequence are required for T cell stimulation.[13]

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[11] See, Van der Zee et al. at page 44, col. 1 "By synthesis of variant peptides evidence was obtained that teach each residue of a seven amino acid long sequence is essential to T cell stimulation."; page 46, col. 1 "Of all nine possible variant peptides (9 amino acids long), full reactivity was only found with peptides having alanine substitutions of residues 187 and 188. All substitutions of residues 180 through 186 resulted in a complete loss of T cell stimulation activity."; page 46, col. 2 "Deletion peptides that lacked any amino acid in the 180 to 186 region were unable to stimulate both T cell clones. . . "; page 47, col. 1 "Apparently all residues in the relatively short 180-186 sequence are required . . ."

[12] See, e.g., The American Heritage Dictionary of the English Language: Fourth Edition (2000) "syngeneic: genetically identical or closely related, so as to allow tissue transplant; immunologically compatible."; Merriam-Webster Dictionary (2000) "syngeneic: genetically identical esp. with respect to antigens or immunological reactions."

[13] "In other studies investigating single amino acid substitutions at all positions within a defined sequence critical for proliferation, the replacement of some residues resulted in equal or even higher stimulatory activity...The present study shows that the occurrence of such indifferent residues is not a general feature of T cell epitopes. Apparently, all residues in the relatively short 180-186 sequence are required for association either with the MHC molecules, or with the T cell receptor or with both." (Van der Zee at page 47, col. 1).

In addition, prior to the present invention, it was not appreciated that one would be able to meaningfully detect cytotoxic T cell activation when more than one peptide species having the requisite MHC-haplotype was released and evaluated in a single ("competitive") assay along with other released peptide species. The instant inventor determined that it would be possible to detect activity elicited from individual species even in a pooled fraction of peptides. Thus, unlike Van der Zee wherein a separate assay is conducted for each peptide species detached from individual rods which are arrayed in a microtiter plate pattern[14], by the present invention, a quantity of peptide is released from each of the solid phase supports in the library of oligopeptides.[15] The array format taught by Van der Zee is spatially addressable thus allowing one to immediately know the amino acid sequence of each peptide (without purification or sequencing) based on its respective position or "address" in the array. Therefore, in contrast to the present invention, Van der Zee does not teach or suggest 'competitive evaluation'.

In view of such teachings, one skilled in the art would not be motivated to release quantities of peptides from a plurality of solid phase supports in a library (necessitating simultaneous testing of multiple peptide species), thereby losing one's ability to (i) determine individual stimulatory index values for each of the multiple peptide species simultaneously assayed; and (ii) immediately know the amino acid sequence of the peptide specie(s) generating the observed activity.

2. Van der Zee does not teach detecting cytotoxic T cell activation by evaluating lysis of the antigen presentation means by the activated cytotoxic T cells.

The invention method is directed to the identification of cytotoxic T cell epitopes by evaluating activated cytotoxic T cells. As such, the method requires that the activity of the T cells, elicited upon the formation of a trimolecular complex of an antigen presentation means/a single species of released oligopeptide/a cytotoxic T cell, is cytolytic activity, wherein the activated cytotoxic T cells lyse the antigen presentation means.

Appellant notes that the teachings of Van der Zee do not teach or suggest cytolytic T cell activity or lysis of antigen presentation means. Rather, Van der Zee relies upon the use of a T cell stimulatory (or proliferation) assay, as set forth in Section 2.2, in order to map and characterize the peptides tested.

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[14] "Therefore, in the present study we developed a procedure for the detachment of the peptide from the solid phase using mild conditions after their simultaneous synthesis by the automated PEPSCAN method." (Van der Zee at sentence bridging pages 43-44). Van der Zee describes the PEPSCAN method: "With this easily automatizable procedure small amounts of several hundreds of peptides are simultaneously synthesized on activated polyethylene rods arrayed in a microtiter plate pattern." (Van der Zee at page 43, col. 2).

Appellant does not challenge the usefulness of such stimulatory assays to measure T cell activity. In some embodiments, one may choose to use a stimulatory assay in addition to detecting the cytolytic activity. However, it is required that each peptide specie identified by the methods of this invention be capable of eliciting cytolytic activity when presented by an antigen presenting cell and complexed with a cytotoxic T cell. The Office can readily appreciate that T cell epitopes capable of eliciting lytic activity will have advantages when used in a therapeutic context as compared with T cell epitopes which merely stimulate the production of T cells.

d. <u>There must be a reasonable expectation of success in making the combination of the references</u>

In establishing a proper prima facie case of obviousness, a reasonable expectation of success in making the combination of the references must exist. The Office has erred by failing to present evidence or provide a rationale that addresses the required expectation of success in making the combination as suggested.

Appellant asserts that the Office's failure to produce such evidence stems from the fact that there is no reasonable expectation of success in arriving at the instant invention by combining the cited prior art references. No reasonable expectation of success can exist because the cited references fail to provide all of the elements of the claimed invention.

As presented above, Appellant has demonstrated that the cited prior art does not contain all of the elements of the claimed invention. None of the prior art references, alone or in combination, teaches or suggests any method to identify cytotoxic T cell epitopes wherein each of the assay components is correlated for MHC-haplotype status. Nor do the prior art references teach detecting cytotoxic T cell activation by evaluating lysis of the antigen presentation means by the activated cytotoxic T cells. Appellant asserts that no reasonable expectation of success in combining the references can exist where the required elements of the instant invention are not all present.

Accordingly, the Office has failed to establish this third required element in a proper prima facie case of obviousness. Appellant also notes that this evidence, demonstrating that there was no reasonable expectation of success, may support the conclusion of nonobviousness[16].

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[15] Peptide libraries that are synthesized in array format, such as the library disclosed by Van der Zee et al., are spatially addressable. Distinct from peptide libraries that are 'mixtures', the amino acid sequence of each peptide (by virtue of its position in the array) is immediately known without purification or sequencing.

[16] *In re* Rinehart, 531 F.2d 1048, (Fed. Cir. 1976).

e. Conclusion

Appellant asserts that the Office has erred in applying the Graham factual inquiries and has failed to establish a prima facie case of obviousness. The Office has used impermissible hindsight reconstruction because there is no motivation or suggestion present that would lead one to combine the references. The collection of references fails to teach all the essential elements of the instant invention, which would preclude the combination suggested by the Office. One skilled in the art would not have reasonably expected success in arriving at the instant invention by making the combination because their combination could not have resulted in the claimed invention.

H. Claims Appendix

1. (Previously presented) A method for identifying a cytotoxic T cell epitope comprising the steps in order of:

a) contacting a population of at least two cytotoxic T cells having the same MHC-haplotype restriction with a quantity of molecule released from a solid phase support, wherein said solid phase support is present in

i) a library of molecules, which molecules are attached to a plurality of solid phase supports by a releasable linker, each of said solid phase supports comprising a plurality of identical copies of a single species of molecule, and wherein the structure of the molecule is determinable, which library of molecules contains a conserved structural motif corresponding to a structural motif characteristic of peptides that associate with the MHC-haplotype to which the cytotoxic T cells are restricted; wherein said quantity of released molecule consists of an amount less than the plurality of said single species of molecule attached to said solid support; and

ii) antigen presentation means, which antigen presentation means correspond to the MHC-haplotype to which the cytotoxic T cells are restricted;

wherein the solid phase supports of the library are in separate fractions;

b) detecting cytotoxic T cell activation effected by the formation of a complex of a cytotoxic T cell, a single specie of released molecule, and said antigen presentation means; and

c) determining the structure of said molecule.

2. (Original) The method according to claim 1, wherein the cytotoxic T cells are selected from the group consisting of

a) polyclonal T cells isolated from a site of cytotoxic T cell infiltration from an individual;
b) cells isolated from a site of cytotoxic T cell infiltration from two or more individuals, which two or more individuals share an MHC haplotype;
c) two or more cytotoxic T cell lines; and
d) any combination thereof.

3. (Withdrawn) The method according to claim 2, wherein the site of cytotoxic T cell infiltration is a tumor.

4. (Original) The method according to claim 1, wherein the molecules are peptides.

5. (Original) The method according to claim 4, wherein the peptides comprise subunits selected from the group consisting of glycine, L-amino acids, D-amino acids, non-classical amino acids, and peptidomimetics.

6. (Original) The method according to claim 1, wherein the solid phase support is selected from the group consisting of polystyrene resin, poly(dimethylacryl)amide-grafted styrene-codivinylbenzene resin, polyamide resin, polystyrene resin grafted with polyethylene glycol, and polydimethylacrylamide resin.

7. (Original) The method according to claim 1, wherein the releasable linker releases upon exposure to an acid, a base, a nucleophile, an electrophile, light, an oxidizing agent, a reducing agent, or an enzyme.

8. (Previously presented) The method according to claim 1, wherein the structural motif contained in the library of molecules is selected from the group consisting of LXXXXXXV (SEQ ID NO: 1); RXXXXXX + (SEQ ID NO: 2); X(D, E) XXXXXX(F, K, Y) (SEQ ID NO: 3); RXXXXXXL (SEQ ID NO: 4); X(K, R)XXXXX(L, I) (SEQ ID NO: 5); (M, L)XXXXXXK (SEQ ID NO: 6); EXXXXXX(Y, F) (SEQ ID NO: 7); XPXXXXX(F, H, W, Y) (SEQ ID NO:8); and (L, I)XXXXX(H, K) (SEQ ID NO:9); wherein X indicates any amino acid residue, and + indicates a positively charged amino acid residue.

9. (Previously presented) The method according to claim 4, wherein the number of representative amino acid residues that are incorporated in the peptides of the library is limited.

10. (Original) The method according to claim 9, wherein positively charged amino acid residues are substituted with an amino acid selected from the group consisting of lysine, arginine, and histidine; negatively charged amino acid residues are substituted with an amino acid selected from the group consisting of aspartic acid and glutamic acid; neutral, polar amino acid residues are substituted with an amino acid selected from the group consisting of asparagine, glutamine, serine, threonine, tyrosine, glycine and cysteine; nonpolar amino acid residues are substituted with an amino acid selected from the group consisting of alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, and methionine.

11. (Original) The method according to claim 10, wherein the nonpolar, aromatic amino acid residues are substituted with an amino acid selected from the group consisting of tyrosine, threonine, and tryptophan; and the nonpolar aliphatic amino acid residues are substituted with an amino acid selected from the group consisting of alanine, valine, leucine, isoleucine, proline, and methionine.

12. (Original) The method according to claim 1, further comprising a coding molecule attached to each to each solid phase support of the library, which coding molecule defines the structure of the molecule attached to the solid phase support by the releasable linker.

13. (Original) The method according to claim 12, wherein the coding molecule is selected from the group consisting of a peptide and an oligonucleotide.

14. (Original) The method according to claim 10, wherein the coding molecule is an inert molecular tag that can be decoded by gas-phase chromatography.

15. (Original) The method according to claim 1, wherein the antigen presentation means is selected from the group consisting of a purified MHC class I molecule complexed to $\beta_2$microglobulin; an intact antigen presenting cell; and a foster antigen presenting cell.

16. (Original) The method according to claim 1, wherein the antigen presentation means is a foster antigen presenting cell.

17. (Original) The method according to claim 16, wherein the foster antigen presenting cell lacks antigen processing activity, whereby it expresses MHC molecules free of bound peptides.

18. (Original) The method according to claim 17, wherein the foster antigen presenting cell is cell line 174xCEM. T2.

19. (Canceled)

20. (Previously presented) The method according to claim 1, wherein cytotoxic T cell activation is detected by a method selected from the group consisting of 3H-thymidine incorporation; metabolic activity detected by conversion of MTT to formazan blue; increased cytokine mRNA expression; increased cytokine protein production; and chromium release by target cells.

21. (Original) The method of claim 1, wherein the structure of the molecule is determined by analyzing a portion of the molecule remaining on the solid phase support.

22. (Original) The method according to claim 4, wherein a sequence of the peptide is determined by sequencing a portion of the peptide remaining on the solid phase support.

23. (Original) The method according to claim 12, wherein the structure of the molecule is determined by analyzing the structure of the coding molecule.

24. (Canceled)

25. (Previously presented) The method according to claim 9, further comprising the steps in order of:

a) contacting the population of at least two cytotoxic T cells having the same MHC-haplotype restriction with a quantity of peptide released from a solid phase support, wherein said solid phase support is present in

i) a library of peptides, which peptides are attached to a plurality of solid phase supports by a releasable linker, each of said solid phase supports comprising a plurality of identical copies of a single species of peptide, and wherein the structure of the peptide is determinable, which library of peptides contains a conserved structural motif corresponding to a structural motif characteristic of peptides that associate with the MHC-haplotype to which the cytotoxic T cells are restricted; wherein said quantity of released peptide consists of an amount less than the plurality of said single species of peptide attached to said solid support; and

ii) antigen presentation means, which antigen presentation means correspond to the MHC-haplotype to which the cytotoxic T cells are restricted;

wherein the solid phase supports of the library are in separate fractions;

b) detecting cytotoxic T cell activation effected by the formation of a complex of a cytotoxic T cell, a single specie of released peptide, and said antigen presentation means; and

c) determining the structure of said peptide.

26. (Previously presented) A method for identifying a high affinity cytotoxic T cell epitope comprising:

a) contacting a population of cytotoxic T cells having an MHC-haplotype restriction with a quantity of molecule released from a solid phase support, wherein said solid phase support is present in

i) a library of molecules, which molecules are attached to a plurality of solid phase supports by a releasable linker, each of said solid phase supports comprising a plurality of identical copies of a single species of molecule, and wherein the structure of the

molecule is determinable, which library of molecules contains a conserved structural motif corresponding to a structural motif characteristic of peptides that associate with the MHC-haplotype to which the cytotoxic T cells are restricted, wherein every amino acid corresponding to a representative residue determined according to the method of claim 9 is utilized at the position identified for the corresponding representative residue; and wherein said quantity of released molecule consists of an amount less than the plurality of said single species of molecule attached to said solid support; and

ii) antigen presentation means, which antigen presentation means correspond to the MHC-haplotype to which the cytotoxic T cells are wherein the solid phase supports of the library are in separate fractions;

b) detecting cytotoxic T cell activation effected by the formation of a complex of a cytotoxic T cell, a single specie of released molecule, and said antigen presentation means; and

c) determining the structure of said molecule.

27. (Original) A method of identifying a protein antigen comprising:

a) identifying the cytotoxic T cell epitope of the protein according to the method of claim 25;

b) comparing a sequence of the T cell epitope identified in step (a) with known sequences of proteins; and

c) determining a protein having a sequence corresponding to the sequence of the T cell epitope.

28. (Original) A method of identifying a protein antigen comprising:

a) identifying the cytotoxic T cell epitope of the protein according to the method of claim 26;

b) comparing a sequence of the T cell epitope identified in step (a) with known sequences of proteins; and

c) determining a protein having a sequence corresponding to the sequence of the T cell epitope.

I. Evidence Appendix

1. Geysen et al., 1984, Proc. Natl. Acad. Sci. USA, Vol. 81: 3998-4002.

J. Related Proceedings Appendix

None.

K. Conclusion

Authorization is hereby given to charge the amount of the fee set forth in 37 C.F.R. § 41.20(b)(2) as well as the fee for a Five Month Extension of time to Deposit Account No. 07-1074. No additional fee is deemed necessary in connection with the filing of this communication. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 07-1074.

Respectfully submitted,

2/21/2006
Date

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Biochemistry

Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid

(antigenic determinant/foot-and-mouth disease virus)

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ABSTRACT **A procedure is described for rapid concurrent synthesis on solid supports of hundreds of peptides, of sufficient purity to react in an enzyme-linked immunosorbent assay. Interaction of synthesized peptides with antibodies is then easily detected without removing them from the support. In this manner an immunogenic epitope of the immunologically important coat protein of foot-and-mouth disease virus (type O_1) is located with a resolution of seven amino acids, corresponding to amino acids 146–152 of that protein. Then, a complete replacement set of peptides in which all 20 amino acids were substituted in turn at every position within the epitope was synthesized, and the particular amino acids conferring specificity for the reaction with antibody were determined. It was found that the leucine residues at positions 148 and 151 were essential for reaction with antisera raised against intact virus. A lesser contribution was derived from the glutamine and alanine residues at positions 149 and 152, respectively. Aside from the practical significance for locating and examining epitopes at high resolution, these findings may lead to better understanding of the basis of antigen–antibody interaction and antibody specificity.**

Recombinant DNA technology now makes possible by deduction from the determined nucleotide sequences reliable amino acid sequences of biologically important proteins. However, methods for identifying the loci in a protein that constitute the antigenic and immunogenic epitopes are few and time consuming and form the bottleneck to further rapid progress. Immunogenic epitopes are defined as those parts of a protein that elicit the antibody response when the whole protein is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule (1–3). On the other hand, a region of a protein molecule to which an antibody can bind is defined as an antigenic epitope. Antisera prepared against chemically synthesized peptides corresponding to short linear tracts of the total polypeptide sequence have been shown to react well with the native protein (4–9). However, interactions were also found to occur even when the site of interaction did not correspond to an immunogenic epitope of the native protein. This has been interpreted to mean that the number of immunogenic epitopes of a protein is less than the number of antigenic epitopes (4). Conversely, since antibodies produced against the native protein are, by definition, directed to the immunogenic epitopes, it follows that peptides reacting with these antibodies must contain elements of the epitopes. From a study of the few proteins for which the determinants have been accurately mapped, it is postulated that a determinant may consist of a single element (continuous) or of more than one element brought together from linearly distant regions of the polypeptide chain by the folding of that chain as it exists in the native state (discontinuous) (10). Systematic mapping of all the detectable reactive elements of a protein by the chemical synthesis of overlapping segments has until now been severely limited by the scale of the synthetic and testing capability required (10, 11). Smith and co-workers (12, 13) circumvented the decoupling and purification steps by combining solid-phase peptide synthesis and solid-phase radioimmunoassay using the same solid support.

We describe here the concurrent synthesis of all 208 possible overlapping hexapeptides covering the total 213-amino acid sequence of the immunologically important coat protein (VP1) of foot-and-mouth disease virus (FMDV), type O_1 (Fig. 1). The peptides, still attached to the support used for their synthesis, were tested for antigenicity by an ELISA using a variety of antisera. After identification of a hexapeptide reactive with antibody raised against the intact virus, all 120 hexapeptides representing the complete single point amino acid replacement set were synthesized and tested for retention of antigenicity. By this method a whole virus epitope was examined at a resolution of a single amino acid.

MATERIALS AND METHODS

Synthesis of Peptides. Polyethylene rods (diameter, 4 mm; length, 40 mm) immersed in a 6% (vol/vol) aqueous solution of acrylic acid were γ irradiated at a dose of 1,000,000 rads (1 rad = 0.01 gray) (15). Rods so prepared were assembled into a polyethylene holder with the format and spacing of a microtiter plate. Subsequent reactions at the tips of the rods were carried out in a Teflon tray with a matrix of wells to match the rod spacing. Conventional methods of solid-phase peptide chemistry (16, 17) were used to couple N^{α}-*t*-butyloxycarbonyl-L-lysine methyl ester to the polyethylene/polyacrylic acid via the N^{ϵ}-amino group of the side chain. Carboxy substitution of the support was determined by treating NH_2-lysine(OMe)-polyethylene/polyacrylic acid with ^{14}C-labeled butyric acid and was found to be 0.15–0.2 nmol/mm^2. Removal of the *t*-butyloxycarbonyl group was followed by the coupling of *t*-butyloxycarbonyl-L-alanine to complete a peptide-like spacer. Successive amino acids were added as dictated by the sequence to be synthesized. At the completion of the final coupling reaction, and after removal of the *t*-butyloxycarbonyl protecting group, the terminal amino group was acetylated with acetic anhydride in dimethylformamide/triethylamine. All *N*,*N*-dicyclohexylcarbodiimide-mediated coupling reactions were carried out in dimethylformamide in the presence of *N*-hydroxybenzotriazole. The following side-chain protecting groups were used: *O*-benzyl for threonine, serine, aspartic acid, glutamic acid, and tyrosine; carbobenzoxy for lysine; tosyl for arginine; 4-methylbenzyl for cysteine; and 1-benzyloxycarbonylamido-2,2,2-trifluoroethyl for histidine. Side-chain-protecting groups were removed by treatment with borontris(trifluor-

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Abbreviations: FMDV, foot-and-mouth disease virus; P_i/NaCl, phosphate-buffered saline.

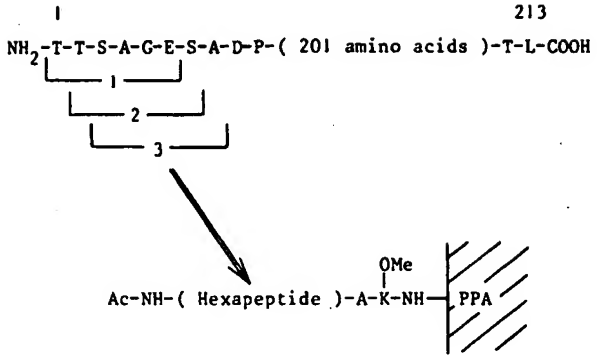

FIG. 1. The 213-amino acid sequence of VP1 (FMDV, type O_1) as translated by Kurz *et al.* (14) was subdivided into hexapeptide units, and each was synthesized on a separate polyethylene support in the orientation, and with a dipeptide spacer, as shown. Peptides are numbered according to the position of the NH-terminal amino acid within the VP1 sequence. PPA, polyethylene/polyacrylic acid.

acetate) in trifluoroacetic acid for 90 min at room temperature (18). After hydrolysis with HCl/propionic acid, sequences included in the synthesis as controls were analyzed to confirm that, although coupling at each stage had occurred, it was incomplete for several of the amino acids, notably arginine. Before testing by ELISA, support-coupled peptides were washed several times with phosphate-buffered saline (P_i/NaCl).

Antisera. Antisera against the intact virus particle were prepared by immunizing rabbits with 50 μg of inactivated, density gradient-purified virus in complete Freund's adjuvant. The animals were bled 3–4 weeks after the single inoculation. Anti-virus-subunit serum was prepared by inoculating rabbits three times, 3–4 weeks apart, with 10 μg of acid-disrupted purified virus, initially in complete Freund's and subsequently in incomplete Freund's adjuvant. The polypeptide VP1 was separated from the mixture of proteins obtained from urea-disrupted purified virus by isoelectric focusing (19). It was eluted from the gel with 8 M urea and dialyzed against P_i/NaCl, and antiserum was raised in rabbits as described for the virus subunit. Antiserum for scan 3 (see Fig. 2) was that used for scan 2 after absorption with purified virus (1500 μg of whole virus was incubated with 1 ml of serum for 72 hr at 4°C), and all virus-bound antibodies were removed by centrifugation.

ELISA. Support-coupled peptides were precoated with 10% horse serum/10% ovalbumin/1% Tween 80 in P_i/NaCl for 1 hr at 37°C to block nonspecific absorption of antibodies. Overnight incubation at 4°C in a 1:40 dilution of antiserum in the preincubation mixture was followed by three washes in 0.05% Tween 80/P_i/NaCl. Reaction for 1 hr at 37°C with a 1:50,000 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase in the preincubation mixture was again followed by extensive washing with P_i/NaCl/Tween to remove excess conjugate. The presence of antibody was detected by reaction for 45 min with a freshly prepared developing solution (40 mg of *o*-phenylenediamine and 20 μl of hydrogen peroxide in 100 ml of phosphate buffer, pH 5.0), and the color produced was read in a Titertek Multiskan (Flow Laboratories, Melbourne, Australia) at 420 nm. Prior to retesting, bound antibody was removed from the peptides by washing peptides three times at 37°C in 8 M urea/0.1% 2-mercaptoethanol/0.1% sodium dodecyl sulfate and then several times with P_i/NaCl.

RESULTS

Identification of a Virus Particle-Associated Immunogenic Epitope. All 208 possible hexapeptides from the amino acid sequence of the VP1 protein of FMDV type O_1 were synthesized in duplicate. The amino acid sequence had been deduced from the nucleotide sequence of the VP1 gene (14). The results obtained for all the synthesized hexapeptides when tested by ELISA with six different antisera are shown in Fig. 2. Antisera used in the test were as follows: two different anti-(intact virus, type O_1), a virus-absorbed anti-(intact virus, type O_1), an anti-(virus subunit, type O_1), an anti-(isolated virus protein VP1, type O_1), and, as a control, an anti-(intact virus, type C_3). The two anti-intact virus sera tested, scans 1 and 2, show the extremes in the reactivity patterns found. Large quantitative differences in the individual animal responses to an identical antigen preparation have been reported before, but these scans highlight the variability possible in the antibody composition between sera. Examination of scans 1, 2, and 3 shows that antibodies reactive with hexapeptide numbers 146 and 147 are present in anti-intact particle sera (scans 1 and 2) but completely absent after absorption of the sera with purified virus (scan 3). Presumably, scan 3 registers those antibodies raised against epitopes expressed in denatured virions that are not present on the surface of the intact virion. Activities to hexapeptides 146 and 147 were not observed in the anti-subunit serum (scan 4) and were only weakly present in the anti-VP1 serum (scan 5). That some activity was found in the anti-VP1 serum

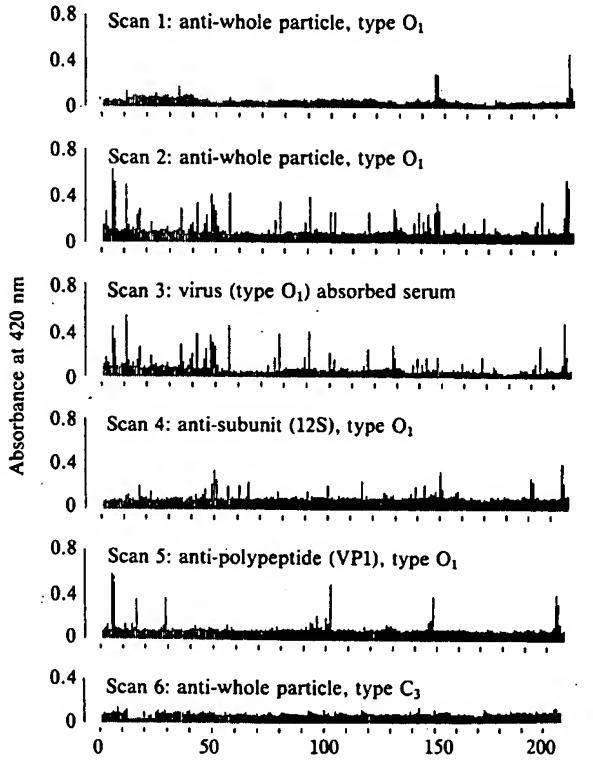

FIG. 2. Antigenic profiles (scans). Results are shown as vertical lines proportional to the extinction obtained in the antibody-binding ELISA test, plotted above the number giving the location within the VP1 sequence of the NH_2-terminal amino acid of each peptide. Antisera used to produce the scans shown were as follows: 1 and 2, two different anti-whole virus particle, type O_1; 3, anti-whole virus particle (as used in 2) after absorption with purified intact virus; 4, anti-virus subunit, type O_1; 5, anti-VP1, type O_1; 6, anti-whole virus particle, type C_3. It should be noted that, because the sequence of VP1 contains 20 alanine residues, 20 of the peptides synthesized match for seven amino acids. However, the frequency of reactive peptides from this group was not significantly different from the overall frequency (0.2 compared with 0.16) and therefore not considered further.

possibly accounts for the immunizing capacity, albeit weak, of the isolated protein (20). It should be noted however that another anti-VP1 serum tested, while retaining a strong activity at position 148, showed no activity at positions 146 and 147. Comparison of scan 3 with scan 2 (absorbed compared with nonabsorbed) shows that, in addition to the loss of activity to peptides 146 and 147, some reduction in activity to peptides 5, 6, and 206 also occurred. Of these, activity to 5 and 6 was not found in all the anti-intact virus sera tested, but activity to 206 was invariably present. From this we conclude that of the peptides found to be reactive, the pair at 146 (G-D-L-Q-V-L) and 147 (D-L-Q-V-L-A) [in this paper, amino acids are identified by the single-letter code (21)] constitute or are part of the principal immunogenic epitope, with the element at 206 (V-A-P-V-K-P) contributing to a lesser epitope. This is consistent with the observations of others (5, 22). Scan 6 shows the absence of reactivity in an antiserum produced against a different serotype of the virus.

Extending the Resolution of the Epitope at Peptides 146/147 to a Single Amino Acid. From the preceding data, we were unable to distinguish between two possibilities: (*i*) the epitope is contained in the five amino acids common to peptides 146 and 147—i.e., D-L-Q-V-L—or (*ii*) the epitope is represented by the "sum" of the two hexapeptides—i.e., G-D-L-Q-V-L-A. To extend the resolution, all 120 possible hexapeptides differing from peptide 146 (G-D-L-Q-V-L) by only a single amino acid were synthesized. Each of the other 19 common amino acids was substituted in each of the six amino acid positions within the peptide. Positions at which all or at least the majority of substitutions result in a loss of antibody-binding activity indicate those residues that are important for the specificity and binding to antibody. The ELISA activity obtained for each of the 120 peptides when serum 48 (anti-intact virus particle) was used in the test are shown in Fig. 3. The relative activities (with respect to the parent sequence) determined for each peptide for two different anti-intact virus sera, nos. 31 and 48, are given in Table 1. To determine the contribution of the alanine residue (carboxyl terminus of peptide 147) toward reactivity and/or specificity, a further 20 peptides were synthesized. Each of these peptides consisted of the complete sequence of 146 (G-D-L-Q-V-L) with one of the 19 possible amino acids added to the carboxyl terminus and synthesized as described before. When serum 31 was used in the test, activity was retained for seven of the amino acids. Relative values expressed in the same way as given in Table 1 were as follows: A (parent amino acid), 99; D, 55; E, 36; G, 45; N, 95; Q, 98; S, 44. With serum 48, activity was retained for four amino acids: A (parent amino acid), 94; G, 30; S, 47; T, 39.

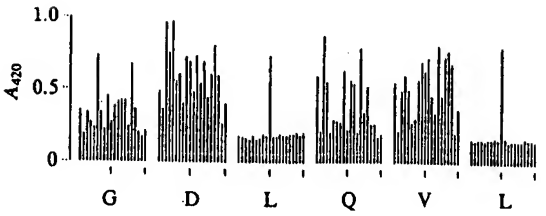

FIG. 3. Antibody-binding activity. The result for each peptide is shown as a vertical line proportional to the ELISA extinction obtained. Every group of 20 lines corresponds to the complete replacement set for one of the six amino acid positions in the hexapeptide G-D-L-Q-V-L. Within each group of 20 lines, the left-hand line corresponds to the substitution of the original residue by alanine (A), and the successive lines are then in alphabetic order according to the single letter code for the amino acids.

DISCUSSION

Interpretation of Data. In choosing to adopt the procedure for peptide synthesis as described, we made several assumptions.

1. To detect antibodies, the quantity of peptide of a defined sequence need only be in the pmol range (5). Assuming a worst-case overall yield of 1% for an eight-step synthesis (two linking and six sequence amino acids), an initial level of 1 nmol of reactive group per support would satisfy the above condition.

2. High purity for the peptide used in the detection of antibodies is not a necessary condition. The majority of serological tests rely on the specificity of antibodies to detect a given antigen in the presence of large amounts of irrelevant protein.

3. Except for cases in which either all or none of the peptides react, a large number of the peptides would effectively act as negative controls in the test. With adjacent peptides sharing a common sequence of five amino acids, the observation of peaks above a generally uniform background level would indicate a valid test.

4. Many of the antibodies elicited by immunization with an intact virus result from presentation of epitopes in fully or partially denatured form. Such antibodies may bind to synthetic peptides *in vitro* but not to the virion itself. They are therefore assumed to be less relevant to virus neutralization

Table 1. Relative antibody-binding activities of peptides derived from the parent sequence G-D-L-Q-V-L

Serum	Parent residue	Activity when substituted with amino acid																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
31	G	29					**90**	14	27	12		32	34	41	29		50				
	D	22	21	**143**	95	110	28	65		12	65	15	58	10	69		38	62			
	L										**79**										
	Q			64	14								13		**80**						
	V	62		33	52				26		29	59			45		49	43	**89**		
	L										**119**										
48	G	11					**88**	10		32		18	24	25	26		77	14			
	D	37	12	**136**	92	137	52	62	21	87	81	37	89	49	80	29	63	104	60		21
	L										**88**										
	Q	60		117	52					68		53	49		**102**	10	45				
	V	52		40	63	42			56	82	68	88	34		106	33	91	98	**81**		14
	L										**105**										

Antibody-binding activities are shown for all peptides that gave an extinction significantly above background. Values for each peptide are expressed as a percentage of the mean activity of the six parent sequences synthesized as a part of each replacement set. Values given boldface type correspond to those obtained for the parent sequence. No activity was detected when the antiserum used was prepared against the heterologous FMDV type.

than are antibodies that bind to virions as well as to peptides.

The extinction obtained in an ELISA for a given peptide depends on the concentration of the antibody population with the correct specificity for reaction. It is essentially independent of the peptide density expressed as reacting groups per mm^2 of support (unpublished data). The difference in the extinction obtained with peptides synthesized with densities varying over two orders of magnitude is similar to the 10–30% variation observed between replicate synthesis (unpublished data). The extinction may also be expected to depend greatly on the affinity between peptide and reacting antibody, but this remains to be verified, although the overnight reaction would tend to minimize differences. Antigenic profiles of the FMDV VP1 (Fig. 2) were interpreted to define an antigenic peptide as one giving an ELISA extinction significantly above the background level of the test. On the other hand, in the testing of replacement nets (Fig. 3), the concentration of the reactive antibody population is constant and effectively of one specificity. Therefore, the extinctions observed are interpreted to reflect the mean affinity of the reacting antibody population for the peptide.

Immunogenicity of FMDV Virus Protein (VP1). Scan 5 of Fig. 2 identifies six immunogenic regions defined in terms of epitopes on the isolated protein eliciting antibodies capable of binding to the corresponding synthetic peptide. Scan 4 shows that, for the same protein as a part of the virus subunit, additional regions (principally 50–70 and 191–197) are immunogenic. Scan 2 shows that, during the course of the immune response to whole virus, most of the protein can be immunogenic. In contrast, scan 1 shows a response to only a very limited number of epitopes. What has become clear from these and other results (unpublished) is that different animals do not necessarily respond to all of the epitopes on a given antigen. In addition, the immunogenic response of an individual animal will be complicated if the antigen is readily broken down as is known to happen to FMDV (23, 24). The animal is exposed not only to the intact virus but also to subunits and possibly even to the isolated viral proteins. Each of these different states could present different epitopes to the immune system. Epitopes can be identified with a particular state of the antigen by testing the peptides with antisera specific to that state.

An Immunogenic Epitope at High Resolution. Antibodies raised against a particular immunogenic epitope will have a combining site (paratope) complementary to the structure of that epitope. An antibody population directed to the same epitope (allowing for variation in the expression of antibodies by the immune reponse) will have common features in the combining sites essential for binding to that epitope. A peptide that, in one of its many conformations in thermal equilibrium *in vitro*, has a structure sufficiently similar to the form of the epitope against which antibody was raised *in vivo* will bind to the antibody. Modification of a reacting peptide by amino acid substitution will define the limits for interaction with antibody. By so "mapping" the antibody-combining site, it is possible to infer properties of the antigen to which this antibody population is complementary. Using polyclonal antisera, it was not expected that a rigorous requirement for particular amino acids in particular positions would be observed. It is clear that, whatever the diversity of the antibodies involved in the interaction, the requirement for a given amino acid in certain positions is absolute for most or all of the antibodies present. It is also clear that the specificity range found for the two different antisera is remarkably similar, differing mainly in the hierarchy of preference for amino acids at the nonessential position. As judged from the limitation to replacements at some position within the sequence G-D-L-Q-V-L-A, the whole-virus epitope may be considered to be X-X-L-Q-X-L-A, where X is nonessential, letters in boldface type indicate an absolute requirement, and letters in lightface type indicate a contributing amino acid.

These findings suggest a different interpretation of the characteristics of epitopes. The antigenic specificity of the epitope represented by amino acids 146–152 within the VP1 protein of FMDV is largely dependent on the leucine residues at positions 148 and 151. These are hydrophobic residues and would not normally be expected to protrude from the protein surface. This suggests the possibility that the immune system responds to a local protein conformation that is different from that expected to represent the global energy minimum. The energy for antigen–antibody binding may be derived from the positive entropy term associated with the transfer of hydrophobic residues from a hydrophilic (aqueous) environment to within the antibody-combining site.

Scope of the Described Approach to Epitope Mapping. Although our results have been presented for a single protein only, the agreement with results of others in locating a viral epitope within the region encompassing amino acids 141–160 of VP1 is excellent (5, 22). The further resolution obtained by Rowlands *et al.* (25) from the comparison of the sequences of the VP1s of three antigenic variants of a single virus type (A_{12}) showed that amino acid substitution at positions 148 and/or 153 would affect the ability to react with specific antibody. This result is in good agreement with our results for subtype O_1, where positions 148 and 151 were critical to the immunogenicity of the epitope. We expect that the systematic approach as outlined, when applied to a broader spectrum of proteins, will contribute greatly to our understanding of the nature of epitopes and their interaction with the immune system.

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